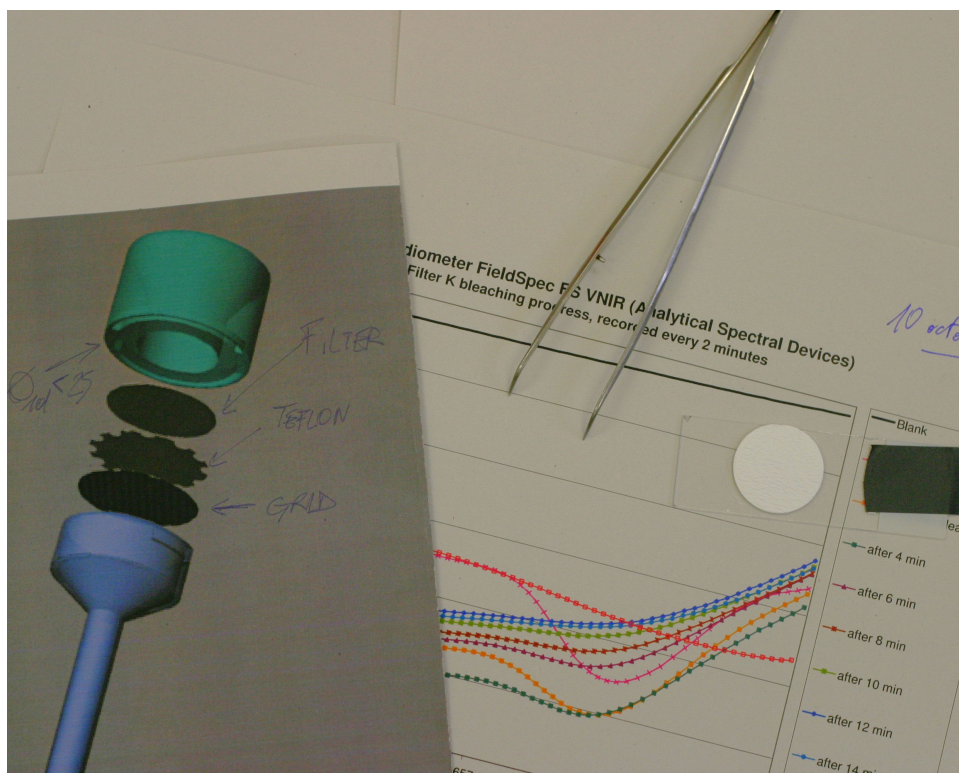


Measuring the absorption coefficient of aquatic particles retained on filter using a photo-oxidation bleaching technique

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Abstract

This report describes a method for the determination of the particulate absorption coefficient that aims to improve the T-R method of Ferrari and Tassan (1995, 2002), giving an alternative to the visual determination of the bleached state of the sample on the filter and introducing a photooxidation bleaching technique to optimize the depigmentation process.

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1. Introduction

Since 1995 a comprehensive data set of atmospheric and marine measurements has been collected in different coastal environments, such as the Adriatic Sea, the Baltic Sea, the North Sea and the English Channel to support oceans color research and development activities. Several in-water inherent optical properties are measured, among these the absorption coefficient of particulate matter, which consists mainly of phytoplankton, and inorganic and organic detritus. The particulate absorption coefficient of phytoplankton pigment and detritus is obtained using spectral transmission and reflection measurements performed before and after the bleaching of phytoplankton pigments by NaClO oxidation (Tassan and Ferrari, 1995, 2002; Ferrari and Tassan, 1999).

The proposed method aims to further on increase the accuracy of the method of Tassan and Ferrari (2002), and gives an alternative solution to the visual determination of the bleached state of the sample filter and to avoid successive bleaching trials.

In particular some operative solutions are introduced to:

- Reduce the loss of material on the sample filter during the depigmentation process (NaClO addition and filter washing)
- To monitor the phytoplankton depigmentation progress during the bleaching treatment by measuring the sample filter reflectance at 675nm by means of a spectroradiometer.
- To optimize the depigmentation process by combining the oxidation carried out by NaClO with a light bleaching action.
- To minimize the undesired bleaching of the colored detritus component present on the sample filter.

2. Materials and Methods

The method used for measuring *in-vivo* light absorption, $a_p(\lambda)$, by aquatic particles retained on filters is detailed in Tassan and Ferrari (1995, 2002) and Ferrari and Tassan (1999). The deposition of particles on filters is obtained by filtration of seawater samples under low vacuum (less than 120 mmHg) to prevent particle breakage and pigment degradation. The samples are filtered through a 25mm diameter glass fiber filters (GF/F, Whatman, Germany) with a 21mm diameter clearance area (Ferrari and Tassan, 1995; Mitchell, 1990; Mitchell et al., 2003; Zibordi et al., 2002) and a nominal pore size of 0.7 μ m in agreement with JGOFS (1991) recommendations. The filtered volumes vary as a function of the quantity of material suspended at the sampling site. After filtration, the filters with the retained deposits are immediately placed in a Petri slide and preserved in

liquid nitrogen. The total absorption coefficient of the equivalent particle suspension in the 400-750 nm spectral range (with 1 nm resolution) is computed according to:

$$a_p(\lambda) = 2.3 A_{sus}(\lambda) \frac{F_a}{V_w}$$

where V_w is the volume of the filtered in water in liters, F_a is the filter clearance area in square centimeters, and $A_{sus}(\lambda)$ is the equivalent particle suspension absorbance obtained from the Transmission and Reflection (T-R) method proposed by Tassan and Ferrari (1995), which has been shown to be appropriate for the analysis of water samples characterized by highly backscattering mineral particles or by highly absorbing sediments. The $a_p(\lambda)$ coefficient is determined with a Perkin Elmer (Fremont, California) Lambda 19 dual-beam spectrometer equipped with a 60 mm diameter integrating sphere.

The method here proposed introduces some modification mainly on the depigmentation procedure of the Tassan-Ferrari methodology. In the original method (Tassan and Ferrari, 1995, 2002; Ferrari and Tassan, 1999) the discrimination due to the pigmented, $a_{ph}(\lambda)$, and the non-pigmented, $a_{dp}(\lambda)$, fraction of the particle absorption coefficient, is obtained by bleaching the sample on the filter by adding a solution of sodium hypochlorite (NaClO) as an oxidizing agent. The filter is positioned horizontal against a 1-mm-thick quartz backing plate and few drops of a 3.3% vol. of NaClO (4% active Cl) in Milli-Q water solution is added as bleaching agent. The operator check visually the bleaching effect of the solution on the filter, when the filter is supposed to be depigmented, it is washed by adding 10 mL of the Milli-Q water under vacuum suction. The sample is analyzed with the spectrophotometer. The effectiveness of the disappearance of the 675 nm peak can be considered evidence of a complete bleaching. If a peak at 675 nm is still present, the NaClO solution adding procedure is repeated until complete depigmentation is observed. The visual determination of the bleached state of the sample on the filter is eliminated in the method here proposed, by introducing a spectroradiometer for monitoring the bleaching progress continuously.

3. Results and Discussion

A series of experiments were carried out to investigate solutions reducing some method uncertainties and optimizing the depigmentation procedure.

The possible resuspension and redistribution of the particles on the filters by adding the oxidizing solution is one of the uncertainties of the methodology. A series of tests were made on real sample replicates by adding the hypochlorite solution in different ways to investigate these effects. The NaClO solution was first dripped at different flow rates by using a volumetric pump. These tests showed that the bleaching acts more

uniform (without showing any “bleach spot” on the filter) when the NaClO solution forms a film on the filter surface. In other experiments the samples were positioned in a filter holder overhead by a funnel (modified 25mm Polysulfone Filter Funnel, PALL, USA) and the oxidizing solution was spiked on the funnel wall. The purpose of the funnel is gentle conveying the NaClO bleaching solution on the filter, avoiding any eventual particle resuspension caused by mechanical impact of the solution's drops on the filter.

The presence of a film on the filter lets the solution penetrate between the different filter fibers, where particles could be trapped, and avoids fast evaporation which occurs with single solution drops after the contact with the filter surface. The persistence of the NaClO on the filter is increased by placing a star shaped Teflon disk (20.5 $\text{\AA}_{\text{o.d.}}$ mm; 25.5 $\text{\AA}_{\text{i.d}}$ mm; thickness: 0.3 mm) under the filter on the filtration grid (grid diameter: 21.5 mm). The Teflon disk avoids the NaClO to pass the filter by gravity. At the same time, it allows for eliminating the NaClO at the end of the bleaching, by applying a vacuum suction. The minifunnel and the star shaped Teflon disk have now been introduced in the common operating procedure (fig.1).

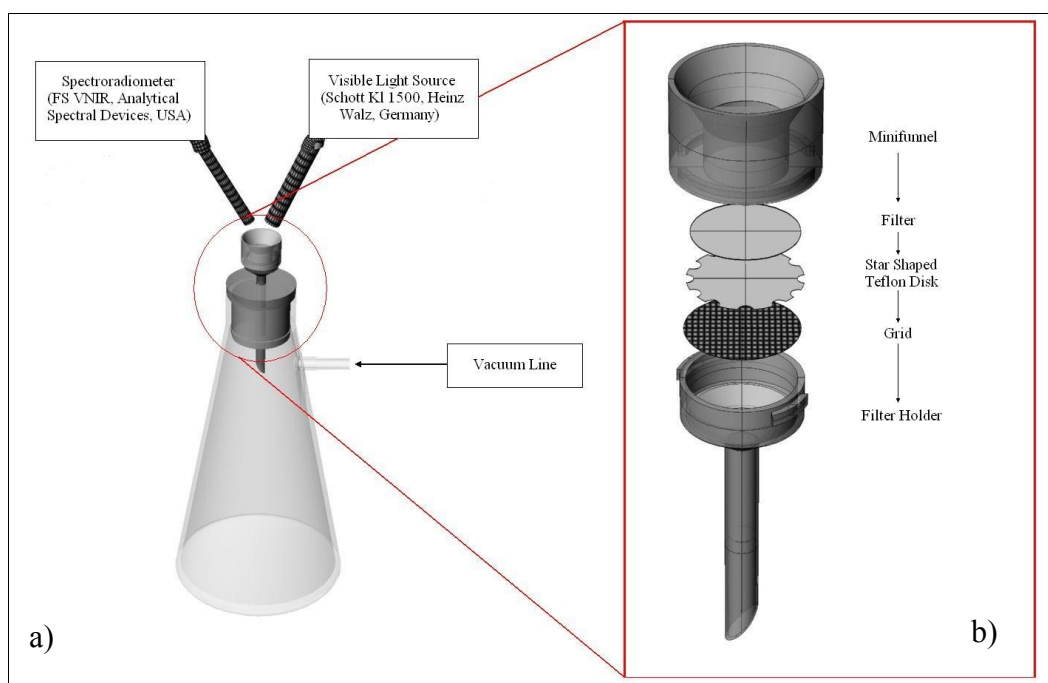


Fig.1: Diagram of **a)** the photooxidation bleaching apparatus realized in order to monitor and control the bleaching progress, and of **b)** the filter washing apparatus.

Through successive trials it was determined that an amount of 1 mL NaClO solution is sufficient to bleach each sample collected in the Adriatic Sea. The use of 1mL NaClO solution is now a standard for the future analysis.

The filter transparency increases with the hydration and, according to Mitchell et al. (2003), before performing measurement in transmittance, the sample filter needs to be wet until fully hydrated by filtered seawater (0.22 μm filter pore size). The same hydration level for all samples was obtained by leaving the filters for circa 30 sec on a sintered disk (Pyrex sintered disk, 50 mm, por.1, Bibby Sterilin, England) immersed in filtered seawater (fig. 2): the sample gets wet by capillarity. This procedure, adopted in the common measurement procedure, avoid the resuspension of the matter on the filter during the hydration step.

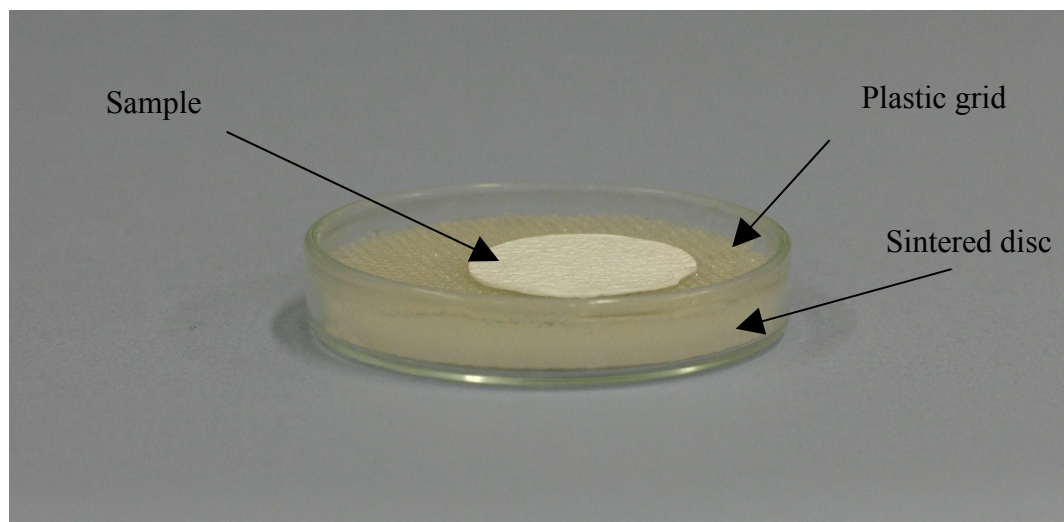


Fig. 2: The sintered disc immersed in the sea water: the samples get wet by capillarity

The necessity of using a light source illuminating the sample during spectroradiometric measurement, suggested to investigate the effect of light on the depigmentation process.

Several studies on solar radiation effects on different phytoplankton species (Häder and Häder 1989 and 1991; Zündorf and Häder, 1991) confirmed the relevant contribution of the visible radiation to the pigments photobleaching. Light dependent degradation of phytoplankton was investigated by Nelson (1993). It is recalled that the photobleaching process does not alter the scattering properties of the phytoplankton cells (Neumüller, M. et al., 2002).

Chemical treatments, however, may induce change in particles size, shape and scattering properties (Mitchell et al., 2000). Combining the photobleaching with the oxidation, a bleaching synergy action is obtained (Nelson, 1993).

The main advantage of monitoring the bleaching process is to reduce the chemical stress induced by the oxidation procedure, leaving the sample in contact with the NaClO solution only for the strictly needed amount of time.

The photooxidation-bleaching apparatus (fig. 1) was set up using a Schott KI 1500 (Heinz Walz, Germany) with Halogene Reflector Lamp, TFR Type (fibre optic

lamp type 6423FO, Philips,) as visible light source and a spectroradiometer (FieldSpec FS VNIR, Analytical Spectral Devices, USA) for monitoring the bleaching progress at 675 nm by using the VNIR software (Analytical Spectral Devices, USA). In fact at 675 nm is possible to observe the *Chlorophyll a* bleaching that, according to the literature, is the pigment with the longest bleaching kinetic (Häder and Häder, 1990 and 1991, Zündorf and Häder, 1991; Kleine et al., 1986). It is possible to use the light at two different intensity ranges: High Light (HL), corresponding to 1240 Lux, and Low Light (LL), corresponding to 670 Lux. Figure 3 displays the irradiance value at the sample surface in the 350-850 nm spectral range.

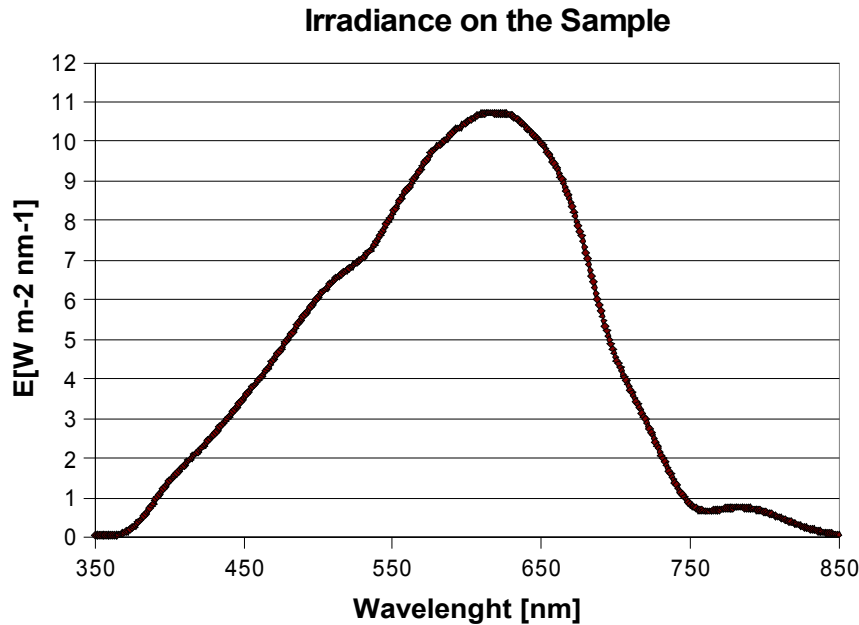


Fig. 3: The irradiance emitted from the visible light source (Schott K1 1500, Heinz Walz, Germany), High Light Intensity (1240 Lux)

In order to identify the optimum operative conditions, tests on real samples replicates were carried out to determine the different contributions to the depigmentation. The samples replicates, series 4, 5 and 6 (consisting of 8 replicates for each serie), were produced at the same time using seawater from the Adriatic Sea. The series 5 and 6 refer to water samples from the surface, while the serie 4 refer to water samples at 14 m depth. The pigments and the TSM characterizing these series, are summarized in table 1.

Series	Depth (m)	Chlorophyllide a	Chlorophyll a	Chlorophyll b	Chlorophyll c1+c2	Fucoxanthin	19'Hex-fucoxanthin
5, 6	0	0.0000	0.6338	0.0403	0.1200	0.0401	0.0782
4	14	0.0000	0.6453	0.0420	0.0987	0.0432	0.07

Series	19'But- fucoxanthin	Diadinoxanthin	Alloxanthin	Diatoxanthin	Zeaxanthin	Beta carotene	Peridinin	TSM (g/m3)
5, 6	0.0094	0.01	0.0192	0.0000	0.0755	0.0242	0.0071	0.77
4	0.0119	0.0070	0.0165	0.0000	0.0386	0.0218	0.0097	0.87

Table. 1: Pigments (mg/m³) present in the series used in the photobleaching tests and the related TSM values.

The sample was positioned in the filter holder on a star shaped Teflon disk. The filter holder was over headed by a funnel (32 Ø_{o.d.} and 19 Ø_{i.d.} mm). The bleaching by oxidation was started gently adding above the sample 1 mL of a solution of 3.3% of NaClO in Milli-Q water. The photo bleaching was started by positioning the sample under the photobleaching apparatus and switching the light on. A black cover was used to protect the sample from ambient light. The parameters considered in the experiments (see Table 2) were: the addition of the oxidation solution, the intensity of the monitoring light radiation and the contribution of the ambient light. For each experiment the time required for a complete depigmentation and the percentage of the effective bleach were recorded.

	Sample (Serie/ Replicates Letter)	Visible Light Source Intensity (Lux)		Measuring Light		Oxidant		Ambient Light Cover		Depigmentation Result	
		1240	670	Constant	At Intervals	NaOCl/ 1ml	No Oxidant (H2O/1ml) *	Yes	No	Minutes	~ %
TEST A: Only Oxidant	4/G	-	-	-	10min	Yes	-	Yes	-	25	95
	5/G	-	-	-	After 30min	Yes	-	Yes	-	30	85
	6/G	-	-	-	After 30min	Yes	-	Yes	-	30	95
	6/H	-	-	-	After 30min	Yes	-	Yes	-	30	95
TEST B: Oxidant + Ambient Light	4/H	-	-	-	10min/ 5min	-	-	-	Yes	20	98
	5/H	-	-	-	10min/ 5min	-	-	-	Yes	30	100
TEST C: Only Visible Light (1240 Light intensity)	4/C	Yes	-	Yes	-	-	Yes	Yes	-	30	95
	4/D	Yes	-	Yes	-	-	Yes	Yes	-	30	90
	5/C	Yes	-	Yes	-	-	Yes	Yes	-	30	95
	5/D	Yes	-	Yes	-	-	Yes	Yes	-	30	90
	6/C	Yes	-	Yes	-	-	Yes	Yes	-	30	85
TEST D: Visible Light (1240 Lux Light intensity) + Oxidant	4/A	Yes	-	Yes	-	Yes	-	Yes	-	10	100
	4/B	Yes	-	Yes	-	Yes	-	Yes	-	10	100
	5/A	Yes	-	Yes	-	Yes	-	Yes	-	10	100
	5/B	Yes	-	Yes	-	Yes	-	Yes	-	10	100
	6/A	Yes	-	Yes	-	Yes	-	Yes	-	10	100
	6/B	Yes	-	Yes	-	Yes	-	Yes	-	10	95
TEST E: Visible Light (670 Lux Light intensity) + Oxidant	4/E	-	Yes	Yes	-	Yes	-	Yes	-	15	100
	4/F	-	Yes	Yes	-	Yes	-	Yes	-	15	100
	5/E	-	Yes	Yes	-	Yes	-	Yes	-	20	95
	5/F	-	Yes	Yes	-	Yes	-	Yes	-	20	95
	6/E	-	Yes	Yes	-	Yes	-	Yes	-	20	95
	6/F	-	Yes	Yes	-	Yes	-	Yes	-	20	90

*1 ml of Milli Q water was added in order to have the same conditions in terms of sample reflectance

Table 2: Summary of the conditions tested for the evaluation of the revised method. The disappearance of the 675 nm peak measured by the FieldSpec FS VNIR spectroradiometer (fig. 4) occurred in a range corresponding to 90-100% of the complete bleaching. The depigmentation of the sample is verified using spectrophotometric measurement on the Lambda 19 (Perkin Elmer)(fig. 5).

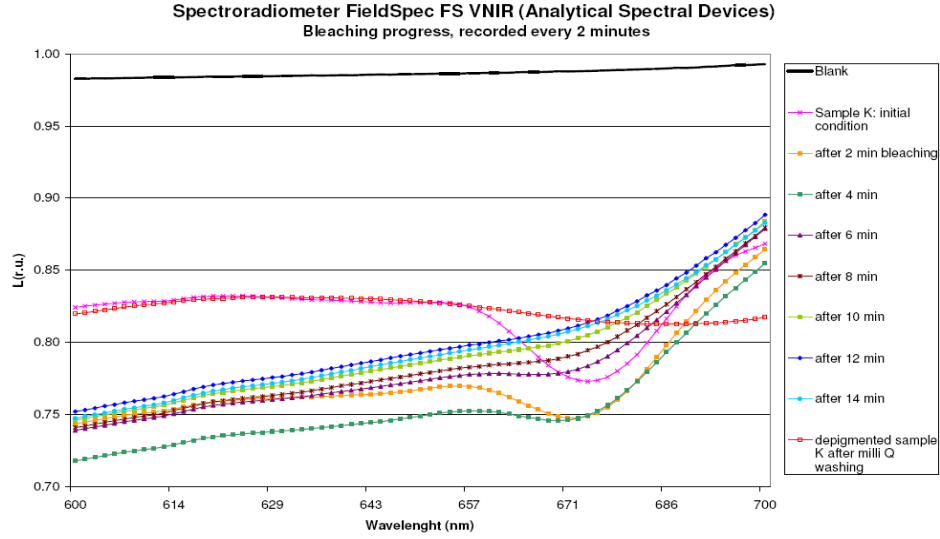


Fig. 4: Radiance from the filter sample k during the bleaching process: the peak at 675 nm is monitored by the spectroradiometer FieldSpec (Analytical Spectral Devices). The spectra were recorded every 2 minutes. The graphic displays a blank and the spectrum of the sample after Milli Q water rinsing.

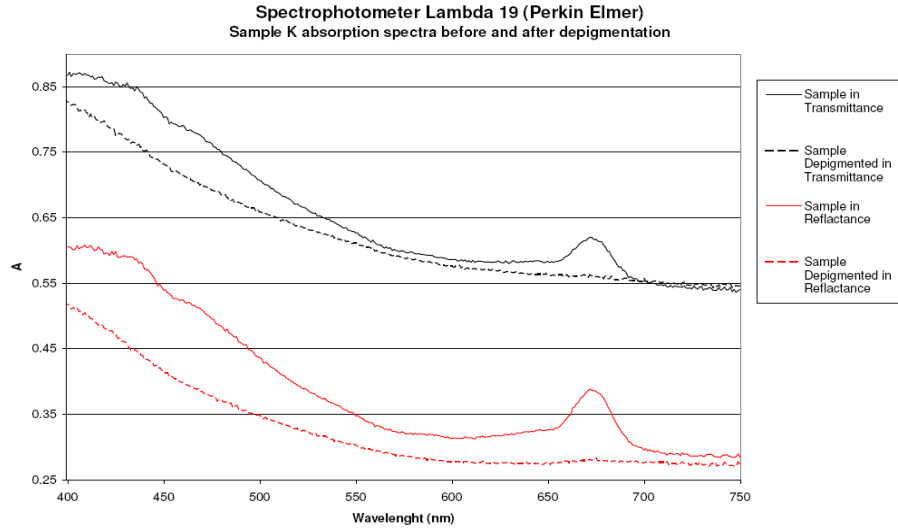


Fig. 5: Absorbance of samples before (continuous line) and after depigmentation (dot line) measured by spectrophotometer Lambda 19 (Perkin Elmer) in Transmittance and in Reflectance modes. The depigmentation procedure was stopped when, according to the operator, the peak at 675 nm, monitored by the spectroradiometer FieldSpec (Analytical Spectral Devices), disappeared.

The two different contributions from the oxidation (TEST A) and photobleaching (TEST C) were estimated. Each process separately needed at least 30 min for a complete

depigmentation. From the TEST D, was evident that the simultaneous presence of photo and oxidation process decrease drastically the depigmentation time (from 30 min required separately to each process to 10 min in synergy). It was indeed observed (TEST D) that the bleaching time decreases as the light intensity increases.

The contribution from ambient light seemed not relevant in the bleaching process (TEST A and B).

The optimum operational condition in terms of bleaching time were obtained by adding 1 mL of a solution of 3.3% vol. of NaClO (4% active Cl) in Milli-Q water on the sample and leaving the light source at High Light (1240 Lux) intensity acting the sample until the bleach is completed.

The consistency of the data was evaluated in term of Absolute Percent Difference (APD) at chosen wavelengths. The APD values obtained comparing results from different experiments made with replicates (see Table 3) are in the expected variation range (see Zibordi et al., 2002).

λ (nm)	412	443	490	510	555	665
ADP % (TEST D vs TEST C)	10.5	11.9	15.4	13.2	4.4	18.7
ADP % (TEST D vs TEST E)	-6.9	-3.5	-9.4	-11.2	-18.9	-9.4

Table 3: APD value determined for the serie 6 of replicates. For each sample the $a_{ph}(\lambda)$ values were normalised on the correspondent $a_p(\lambda)$.

4. Conclusion

The Transmission-Reflection method proposed by Tassan and Ferrari (1995) for measuring *in-vivo* light absorption, $a_p(\lambda)$, still presents, in the revised version (2002), several uncertainties and some aspects not completely clarified, mainly concerning the depigmentation procedure. The method here proposed introduces a new procedure for adding the oxidant solution and for monitoring the depigmentation process with the aims to not alterate particles distribution on the filter and to avoid any unnecessary physic-chemical stress on the filter. The new procedure includes a filtration system apparatus equipped with a spectroradiometer for monitoring the bleaching process constantly. The spectroradiometer monitoring resulted effective on all the considered phytoplankton pigments. The filtration system allows the addition of the oxidant solution and of Milli-Q water preventing the particles washing out from filter. The filter holder lets the oxidation solution forming a film on the filter and acting in the different filter layers. The quantity of the oxidant solution is fixed.

To optimize the depigmentation procedure, the photobleaching contribution in the range of the visible was positively investigated. The photobleaching action, combined with the oxidant action carried on by the sodium hypochlorite, allows for a complete depigmentation of the samples in a considerable shorter time (approximately one third of the time required using the oxidation solution only), reducing at the same time the sample stress.

The filtration system apparatus and all the modifications to the Tassan and Ferrari procedure, including photobleaching, here described are permanently introduced in the routine analysis.

Acronyms and Symbols

APD :Absolute Percent Difference

T-R :Transmission-Reflection (method for spectrophotometric analysis)

TSM :Total Suspended Matter

$a_{dp}(\lambda)$:Non-pigmented particulate matter absorption coefficient

$a_p(\lambda)$:Particulate matter absorption coefficient

$a_{ph}(\lambda)$:Pigmented particulate matter absorption coefficient

$A_{sus}(\lambda)$:Equivalent particle suspension absorbance

F_a :Filter clearance area

V_w :Volume of filtered

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